REMARKS

Claims 8-11 are pending. Claims 1-7 and 12-30 have been cancelled without prejudice.

Claims 10 and 11 have been amended to indicate that the sequences are DNA sequences that are used to synthesize the siRNA molecules. Support for the amendments to claims 10 and 11 can be found in the specification beginning at paragraph [0114] on page 31.

The amendments to the claims are not believed to introduce new matter into the application.

**I. Claims Rejections – 35 U.S.C. § 112**

Claims 8-11 have been rejected under the first paragraph of 35 U.S.C. § 112, as allegedly failing to comply with the enablement requirement. The Office Action alleges that the claims are not enabled because they are directed to siRNA species consisting of particular RNAi sequences (e.g., SEQ ID NO:9 and 10) but the sequences shown are DNA sequences.

The sequences shown are indeed DNA sequences. The shown sequences are DNA templates that were used to synthesize the siRNA molecules using the Ambion, Inc. silencer siRNA construction kit (Specification: paragraph [0114]). The specification beginning at paragraph [0114] teaches that the Ambion, Inc. silencer siRNA kit was used to make the siRNA molecules. Methods for making siRNA molecules DNA templates is well known in the art. A person of ordinary skill in the art would recognize that the sequences shown in the application are DNA sequences and as such, are templates for making the sense and antisense strands of the siRNA molecule. The skilled artisan would also understand the method of the Ambion, Inc, kit and understand how the template molecules are used to make the siRNA molecules.

For example, for the H4 site at position 556, SEQ ID NO:9 is the template for the RNA corresponding to the sense strand overlapping the H4 position and SEQ ID NO:10 is the template for the RNA corresponding to the anti-sense strand overlapping the H4 position. In all of the sequences, the CTCTGTCC sequence is a primer for the T7 RNA polymerase to make the RNA sense and antisense strands.

In the first step, DNA primers containing a T7 polymerase initiation site followed by the complement to the CTCTGTCC sequence at the 3' end are hybridized to each template molecule in a pair at the 8 nucleotide CTCTGTCC sequence to produce double-stranded DNA molecules with an eight-nucleotide hybridized region and long single-stranded, unhybridized strands. Klenow DNA polymerase is then used to make fully double-stranded DNA molecules of each template molecule in the pair.

To make an siRNA molecule, RNA molecules are transcribed from both double-stranded DNA molecules in a pair and the RNA strands hybridized together to form a dsRNA molecule with unhybridized CTCTGTCC leaders at the 5' ends of both strands and unhybridized UU dinucleotides at the 3' ends of both strands. The dsRNA is then digested with a single-strand specific RNAse that can remove the CTCTGTCC leader but not the 3'UU dinucleotide. Using SEQ ID NO:9 and 10 as examples, the resulting double-stranded siRNA molecule expected would be

```
  5'-GAGGGUCUCAGGAGACTGUU -3' sense strand
     | | | | | | | | | | | | | | | | | | |
3'-UUCUCCCAGAGUCCUCUGACA -5' antisense strand
```

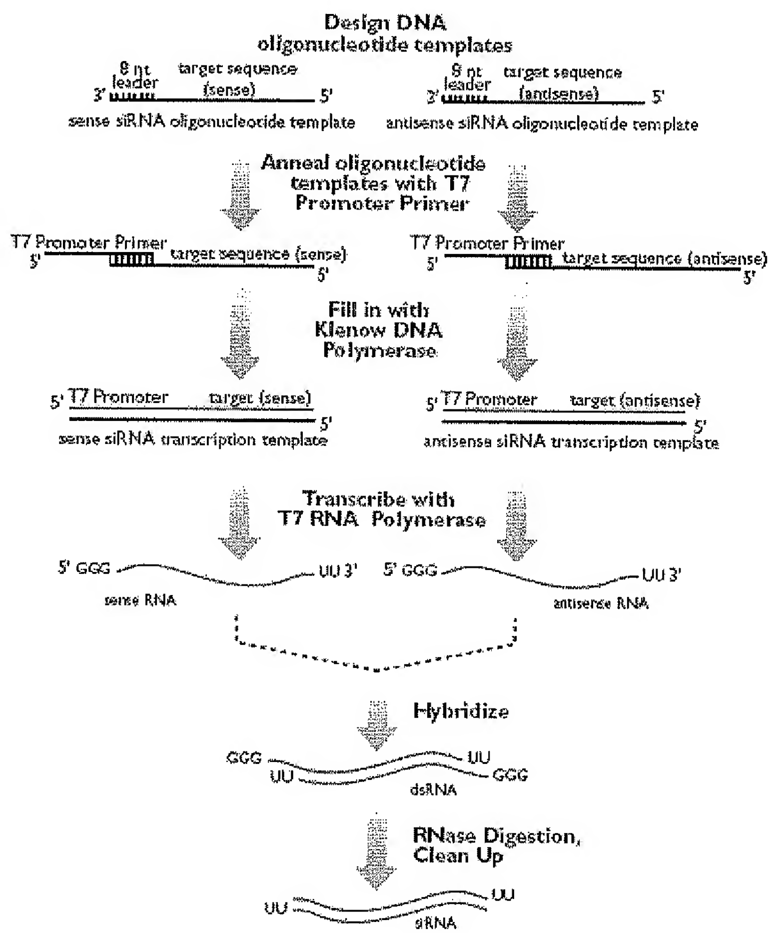
Below are shown the DNA templates with the leader sequence underlined that were used to make the H4 sense and antisense RNA strands comprising the H4 siRNA.

H4 (Position 556)
Sense: AAACAGTCTCCTGAGACCCTC**<u>CCTGTCTC</u>** (SEQ ID NO: 9) [template used to make 5'-GAGGGUCUCAGGAGACTGUU-3' sense strand]

Antisense: AAGAGGGTCTCAGGAGACTGT**<u>CCTGTCTC</u>** (SEQ ID NO: 10) [template used to make 3'-UUCUCCCAGAGUCCUCUGACA-5' antisense strand]

A copy of the instruction manual from Ambion, Inc. describing how to make siRNA molecules from their kit is included herewith as Exhibit A. Below is Figure 1 reproduced from the manual illustrating how the templates shown in the specification and as currently claimed would be used to synthesize siRNA molecules.

Figure 1. *Silencer®* siRNA Construction Kit Procedure




In light of the above, a person of ordinary skill in the art in view of the specification and imbued with the general knowledge in the art of siRNA molecules would have understood that the DNA sequences shown in the application are DNA templates that can be used to produce siRNA molecules. Thus, it is believed that a person of ordinary skill would be able to practice the currently claimed invention without undue experimentation.

Therefore, in light of the above, the currently amended claims are believed to satisfy the enablement requirement of the first paragraph of 35 U.S.C. § 112. Reconsideration of the rejection is requested.

In view of the foregoing remarks and amendments, it is believed that the grounds of rejections have been overcome and that the claims are in proper condition for allowance. Accordingly, Applicants respectfully request that all of the rejections be withdrawn and a Notice

of Allowance be forwarded to the Applicants. The Examiner is invited to contact Applicants' Attorney at the telephone number given below, if such would expedite the allowance of this application.

Favorable action is earnestly solicited.

CONDITIONAL PETITION

Applicant hereby makes a Conditional Petition for any relief available to correct any defect in connection with this filing, or any defect remaining in this application after this filing. The Commissioner is authorized to charge deposit account 13-2755 for the petition fee and any other fee(s) required to effect this Conditional Petition.

Respectfully submitted,

By /John David Reilly, Reg. #43,039/
JOHN DAVID REILLY
Reg. No. 43,039
Attorney for Applicant

MERCK & CO., INC.
P.O. Box 2000
Rahway, New Jersey 07065-0907
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Date: December 1, 2009

EXHIBIT A

# *Silencer*® siRNA Construction Kit

(Part Number AM1620)

*Protocol*

**P/N 1620M Revision C** **Revision Date: August 26, 2008**

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**Literature Citation:** When describing a procedure for publication using this product, please refer to it as the *Silencer*® siRNA Construction Kit.

If a paper that cites one of Ambion's products is published in a research journal, the author(s) may receive a free Ambion T-shirt by sending in the completed form at the back of this protocol, along with a copy of the paper.

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**Patents and Licensing Notifications:** The *Silencer* siRNA Construction Kit is covered by US patents 5256555, 6586218, 6586219 and US and foreign patents pending.

Ambion has been granted rights by the Massachusetts Institute of Technology to US Patent Applications 60/265232, 09/821832 and PCT/US01/10188, RNA Sequence-Specific Mediators of RNA Interference. This product is licensed under European Patents 1144623, 1214945 and foreign equivalents from AlnylamPharmaceuticals, Inc., Cambridge, USA and is provided only for use in academic and commercial research including in vitro and in vivo identification and validation of drug targets (but excluding the evaluation or characterization of this product as the potential basis for a siRNA-based drug) and not for any other commercial purposes. Information about licenses for commercial use (including discovery and development of siRNAbased drugs) is available from Alnylam Pharmaceuticals, Inc., 300 Third Street, Cambridge, MA 02142, USA.

**Trademarks:** Applied Biosystems, AB (Design), Ambion, NorthernMax, RNase*Zap* and *Silencer* are registered trademarks, and RPA III is a trademark of Applied Biosystems Inc. or its subsidiaries in the US and/or certain other countries. All other trademarks are the sole property of their respective owners.

# I. Introduction

## A. Background

Post-Transcriptional Gene Silencing and RNA interference (RNAi) are terms describing the specific suppression of genes by complementary dsRNA (for a recent review, see Sharp 2001). Although the mechanism by which dsRNA suppresses gene expression is not entirely understood, experimental data provide important insights. In non-mammalian systems such as *Drosophila*, it appears that longer dsRNA is processed into 21–23 nt dsRNA (called small interfering RNA or siRNA) by an enzyme containing RNase III motifs (Bernstein et al., 2001, Grishok et al., 2001, Hamilton and Baulcombe 1999, Knight and Bass 2001, Zamore et al., 2000). The siRNA apparently then acts as a guide sequence within a multicomponent nuclease complex to target complementary mRNA for degradation (Hammond et al., 2001).

Long dsRNA is routinely used in non-mammalian cells and organisms to effect gene silencing. Mammalian cells, however, have a potent antiviral response pathway that induces global changes in gene expression when dsRNA molecules longer than 30 nt are introduced into cells (Stark et al., 1998, Manche et al., 1992, Minks et al., 1979). The antiviral response makes it difficult to distinguish target-specific effects of long dsRNA from the general antiviral response. siRNAs, comprised of 21-mer dsRNAs, do not trigger the antiviral response, making it possible to perform gene silencing experiments in mammalian cells (Elbashir et al., 2001, Caplen et al., 2001, Hutvagner et al., 2001, Jarvis and Ford 2001).

**siRNA synthesis by in vitro transcription**

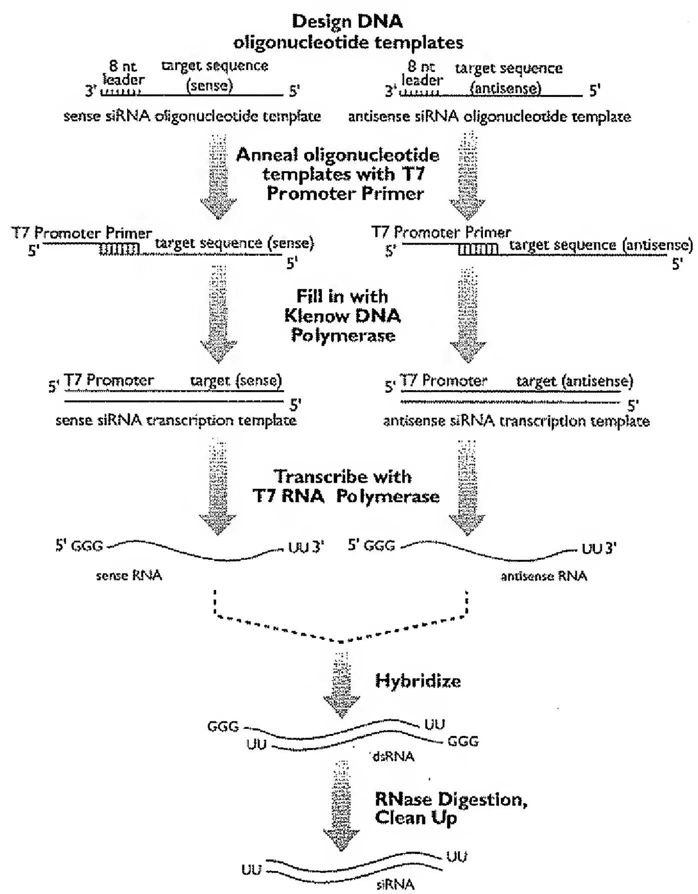
Whereas most RNAi experiments in *Drosophila*, *C. elegans*, and other non-mammalian cells have used in vitro transcribed RNA molecules, early siRNA experiments in mammalian cells used chemically synthesized RNA oligomers. Chemically synthesized siRNAs were probably used because there are no sequence limitations for chemical synthesis. In contrast, in vitro transcription using T7 RNA polymerase requires that the first 2 nucleotides of the RNA transcript be GG or GA to ensure efficient synthesis (Milligan 1987). Requiring a GG or GA at the 5' ends of both the sense and antisense strands of an siRNA in addition to the required 3' terminal UU greatly reduces the number of potential target sites for siRNA experiments. This constraint essentially eliminates in vitro transcription as a viable option for preparing siRNAs.

The *Silencer* siRNA Construction Kit overcomes the sequence requirements of traditional in vitro transcription strategies by using siRNA template oligonucleotides containing a "leader" sequence that is complementary to the T7 Promoter Primer included in the kit (Figure 2). Inclusion of leader sequences provides two benefits:

- The 8 nt leader sequence is optimized for maximal RNA yield.
- After transcription and hybridization of the sense and antisense strands of the siRNA, the leader sequences are efficiently removed from the dsRNA preparation, eliminating the need to select target mRNA sequences that are compatible with T7 transcription.

### *Silencer* siRNA Construction Kit procedure overview

Figure 1. *Silencer®* siRNA Construction Kit Procedure




a. Two 29-mer DNA oligonucleotides (template oligonucleotides) with 21 nt encoding the siRNA and 8 nt complementary to the T7 Promoter Primer are synthesized and desalted.

b. In separate reactions, the 2 template oligonucleotides are hybridized to a T7 Promoter Primer (an oligonucleotide provided with the kit that contains a T7 promoter sequence and 8 nt complementary to the template oligonucleotides).

c. The 3' ends of the hybridized DNA oligonucleotides are extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates.

d. The sense and antisense siRNA templates are transcribed by T7 RNA polymerase and the resulting RNA transcripts are hybridized to create dsRNA. The dsRNA consists of 5' terminal single-stranded leader sequences, a 19 nt target specific dsRNA, and 3' terminal UUs.

e. The leader sequences are removed by digesting the dsRNA with a single-strand specific ribonuclease. Overhanging UU dinucleotides will remain on the siRNA because the RNase does not cleave U residues. The DNA template is removed at the same time by a deoxyribonuclease.

f. The resulting siRNA is purified by glass fiber filter binding and elution which removes excess nucleotides, short oligomers, proteins, and salts in the reaction.

The end product is a double-stranded 21-mer siRNA with 3' terminal uridine dimers that can effectively reduce the expression of target mRNA when transfected into mammalian cells.

## B. Reagents Provided With the Kit and Storage

The *Silencer* siRNA Construction Kit provides reagents for construction of 15 different siRNAs.

### Template Preparation

| Amount | Component | Storage |
|---|---|---|
| 200 µL | DNA Hybridization Buffer | –20°C |
| 60 µL | T7 Promoter Primer | –20°C |
| 60 µL | 10X Klenow Reaction Buffer | –20°C |
| 60 µL | 10X dNTP Mix | –20°C |
| 60 µL | Exo– Klenow | –20°C |

### Transcription Reagents

| Amount | Component | Storage |
|---|---|---|
| 60 µL | T7 Enzyme Mix | –20°C |
| 60 µL | 10X T7 Reaction Buffer | –20°C |
| 300 µL | 2X NTP Mix* | –20°C |

* Kit life can be prolonged by storing the 2X NTP Mix at –80°C

### siRNA Purification

| Amount | Component | Storage |
|---|---|---|
| 90 µL | Digestion Buffer | –20°C |
| 37.5 µL | DNase | –20°C |
| 45 µL | RNase | –20°C |
| 7.8 mL | siRNA Binding Buffer<br>Add 5.3 mL 100% ethanol before use | room temp |
| 20 mL | siRNA Wash Buffer<br>Add 11 mL 100% ethanol before use | room temp |
| 15 | Filter Cartridges | room temp |
| 30 | Collection Tubes | room temp |
| 3.5 mL | Nuclease-free Water | any temp* |

* Store Nuclease-free Water at –20°C, 4°C, or room temp

### Positive Control Reagents

| Amount | Component | Storage |
|---|---|---|
| 10 µL | Sense Control DNA (100 µM) | –20°C |
| 10 µL | Antisense Control DNA (100 µM) | –20°C |

## C. Materials Not Supplied with the Kit

**Template oligonucleotides (DNA)**

Template oligonucleotides (DNA) are 29 nt in length. The 8 nucleotides at the 3' end of each oligonucleotide must be 5'-CCTGTCTC-3'. See section *II.A. siRNA Design* starting on page 7 for complete instructions on designing template oligonucleotides.

Order the smallest scale synthesis of the oligonucleotides. Desalting is typically sufficient purification.

**(optional) Radiolabeled NTP**

Radiolabeled NTP, for example [$\alpha$-$^{32}$P]GTP, ATP, or CTP, can be included in the reaction as a tracer to aid in the quantitation and assessment of the siRNA synthesized. Any specific activity of radiolabeled nucleotide is acceptable.

**IMPORTANT**

*[$\alpha$-$^{32}$P]UTP should not be used to trace label the siRNA because a modified UTP is present in the NTP mixture to enhance siRNA potency.*

## D. Related Products Available from Applied Biosystems

| | |
|---|---|
| RNase*Zap*® P/N AM9780, AM9782, AM9784 | RNase Decontamination Solution. RNase*Zap* is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*. |
| *Silencer*® siRNAs See web or print catalog for P/Ns | Ambion *Silencer* Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer* siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer* siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer* siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects. |
| *Silencer*® siRNA Transfection II Kit P/N AM1631 | The *Silencer* siRNA Transfection II Kit contains both siPORT™ *NeoFX*™ and siPORT *Lipid* Transfection Agents in addition to a well-characterized siRNA targeting human, mouse, and rat GAPDH. This kit is ideal for developing an optimal transfection protocol for your cells. Also included are a highly validated non-targeting negative control siRNA and a detailed Protocol. |
| Negative Control siRNA and Templates See web or print catalog for P/Ns | Universal scrambled siRNA control sequences are available separately as either prepared and tested siRNA or templates for use in the *Silencer* siRNA Construction Kit. The scrambled controls have no significant homology to mouse, rat, or human gene sequences and are ideal for use as negative controls in any siRNA experiment. |
| Antibodies for siRNA Research See web or print catalog for P/Ns | For select *Silencer* Control and Validated siRNAs, Ambion offers corresponding antibodies for protein detection. These antibodies are ideal for confirming mRNA knockdown results by analyzing concomitant protein levels. |

| | |
|---|---|
| NorthernMax® Kits<br>P/N AM1940, AM1946 | Ambion NorthernMax Kits: NorthernMax, and NorthernMax-Gly, combine ultrasensitive, reliable Northern blot protocols with unsurpassed quality control to ensure optimal results in less time. |
| RPA III™<br>P/N AM1414, AM1415 | Ribonuclease Protection Assay Kit for the detection and quantitation of mRNA. This kit incorporates Ambion's exclusive one-tube format, for fast sensitive detection of RNA with no proteinase K or phenol extraction steps. |

# II. *Silencer* siRNA Construction Kit Instructions

## A. siRNA Design

Using siRNA for gene silencing is a rapidly evolving tool in molecular biology; these instructions are based on both the current literature, and on empirical observations by scientists at Ambion. Because we are able to modify information on our web site so quickly (compared to printed documents), you may want to check the "siRNA Design" page on our web site for the latest recommendations on siRNA target selection.
www.ambion.com/techlib/misc/siRNA_design.html

### 1. Find 21 nt sequences in the target mRNA that begin with an AA dinucleotide

Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites.

This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. (EMBO 2001) that siRNA with 3' overhanging UU dinucleotides are the most effective. Since then, however, siRNA with other 3' terminal dinucleotide overhangs have been transfected into cells and shown to induce RNAi. If desired, you may modify this target site selection strategy to produce siRNA with other dinucleotide overhangs, but it is essential to avoid G residues in the overhang because the siRNA will be cleaved by RNase at single-stranded G residues.

### 2. Select 2–4 target sequences

Research at Ambion has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75–95% reduction. Choose target sites from among the sequences identified in step 1 based on the following guidelines:

- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
- Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at:
  www.ncbi.nlm.nih.gov/BLAST.
- Ambion researchers find that siRNAs with 30–50% GC content are more active than those with a higher G/C content.

### 3. Negative Controls

siRNA experiments should include a negative control siRNA with the same nucleotide composition as the experimental siRNA but which lacks significant sequence homology to the genome. To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.

### 4. Design template oligonucleotides (DNA)

**a. See Figure 2 on page 8 for an example of template oligonucleotide design.**

- The *antisense* template oligonucleotide should have 21 nt at the 5' end that is the DNA counterpart of the target mRNA sequence chosen, i.e. the same sequence as the target RNA except that U residues are replaced with T's.
- The *sense* template oligonucleotide should start with an AA dinucleotide at the 5' end followed by 19 nt that are complementary to the target sequence identified in step 2.
- The 8 nt at the 3' end of ***both*** oligonucleotides should be the following sequence: 5'-CCTGTCTC-3'.

  This 8 nt sequence is complementary to the T7 Promoter Primer provided with the *Silencer* siRNA Construction Kit. Hybridization of the template oligonucleotides to the T7 Promoter Primer will add the T7 promoter sequence to the 5' ends of the template oligonucleotide so that after the fill-in reaction, they can be efficiently transcribed.

Figure 2. Example of Template Oligonucleotide Design

Target mRNA sequence

5'-AACGAUUGACAGCGGAUUGCC-3'

Order these oligonucleotides to make an siRNA that targets the mRNA sequence shown above:

Antisense template oligonucleotide (DNA)

5'-AACGATTGACAGCGGATTGCCCCTGTCTC-3'

Sense template oligonucleotide (DNA)

5'-AAGGCAATCCGCTGTCAATCGCCTGTCTC-3'

**b. Check your design**

Note that transcription of the antisense oligonucleotide will generate RNA that is complementary to the target mRNA.

Also note that transcription of the sense template generates a 3' terminal UU that is not complementary to the antisense strand of the siRNA. This UU sequence does not need to be part of the mRNA sequence because the sense strand of the siRNA appears to have no function in targeting mRNAs for degradation.

c. **Order the oligonucleotides**

Order the sense and antisense template DNA oligonucleotides for each siRNA. The smallest scale synthesis (40 nmol or less) is sufficient for hundreds of transcription reactions. Desalting is typically sufficient purification for generating efficient transcription templates.

## B. Transcription Template Preparation

To make an efficient transcription template, the sense and antisense template oligonucleotides (DNA) for each siRNA must be converted to dsDNA with a T7 promoter at the 5' end. This is accomplished by hybridizing the 2 oligonucleotides to the T7 Promoter Primer provided with the *Silencer* siRNA Construction Kit and extending the T7 Promoter Primer and template oligonucleotides using a DNA polymerization reaction.

### 1. Resuspend the template oligonucleotides to 200 µM in nuclease-free water

Oligonucleotides are usually supplied dry; tap the tubes containing the oligonucleotides on the bench to force the powder to the bottom of the tubes. Check the specification sheet supplied with the oligonucleotides to see how much was synthesized, and dissolve the sense and antisense template oligonucleotides in nuclease-free water to approximately 200 µM.

### 2. Determine the template oligonucleotide concentration by $A_{260}$

a. **Measure the $A_{260}$ of a 1:250 dilution of the DNA template oligonucleotides**

Dilute a small sample of the sense and antisense template oligonucleotides 1:250 into TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and read the absorbance at 260 nm in a spectrophotometer. Be sure to blank the spectrophotometer with the same TE that was used for sample dilution.

b. **Determine the oligonucleotide concentration in µg/mL**

Multiply the absorbance reading by 5000 to determine the concentration of the oligonucleotides in µg/mL. (See the explanation below.)

5000 = 250-fold dilution X 20 µg oligo/mL per absorbance unit*

* 20 µg/mL is used to compensate for the non-full length oligonucleotide that is typically present in chemically synthesized oligonucleotide preps.

**c. Determine the molar concentration of the oligonucleotides**

The molar concentration of the oligonucleotides in µM can be determined by dividing the µg/mL concentration by 9.7. (See the explanation below.)

- There are 9.7 µg of DNA in 1 nmole of an average 29-mer:

  29 nt X 0.333 µg/nmol for each nt = 9.7 µg/nmol

- Dividing the µg/mL concentration by 9.7 yields the µM concentration as shown below:

$$\frac{\frac{X\ \mu g}{mL}}{\frac{9.7\ \mu g}{nmol}} = \frac{X\ \mu g}{mL} \times \frac{nmol}{9.7\ \mu g} = \frac{X\ nmol}{mL\ (9.7)} \quad \frac{X\ \mu mol}{L\ (9.7)} \quad \frac{X\ \mu M}{9.7}$$

Therefore µM = X ÷ 9.7

**d. Example calculation**

A 1:250 dilution of an oligonucleotide solution has an $A_{260}$ of 0.4. The molar concentration is determined as follows:

0.4 x 5000 µg/mL per $A_{260}$ = 2000 µg/mL

2000 µg/mL divided by 9.7 µg/nmol = ~206 µM

**3. Make a 100 µM solution of each oligonucleotide**

Dilute an aliquot of each template oligonucleotide to 100 µM using nuclease-free water or TE (10 mM Tris-HCl pH 8, 1 mM EDTA). Prepare ~20 µL of 100 µM oligonucleotide solutions.

**4. Thaw the frozen template preparation reagents**

Thaw the following kit components at room temperature, then briefly vortex each before use.

- T7 Promoter Primer
- 10X Klenow Reaction Buffer
- 10X dNTP Mix
- Nuclease-free Water

**IMPORTANT**

*Keep the tube of Exo– Klenow at –20°C and do not vortex it.*

**5. Hybridize each template oligonucleotide to the T7 Promoter Primer**

a. In separate tubes mix the following:

| Amount | Component |
|---|---|
| 2 µL | T7 Promoter Primer |
| 6 µL | DNA Hyb Buffer |
| 2 µL | *either* sense *or* antisense template oligonucleotide |

b. Heat the mixture to 70°C for 5 min, then leave at room temp for 5 min.

**6. Fill in with Klenow DNA polymerase**

a. Add the following to the hybridized oligonucleotides:

| Amount | Component |
|---|---|
| 2 µL | 10X Klenow Reaction Buffer |
| 2 µL | 10X dNTP Mix |
| 4 µL | Nuclease-free Water |
| 2 µL | Exo– Klenow |

b. Gently mix by pipetting or slow vortexing. Centrifuge briefly to collect the mixture at the bottom of the tube.

c. Transfer to 37°C incubator and incubate for 30 min.

**7. Proceed to dsRNA synthesis, or store the templates at –20°C**

The siRNA templates can be used directly in a transcription reaction (see section *C. dsRNA Synthesis* below) or stored at –20°C until they are needed for transcription.

## C. dsRNA Synthesis

The sense and antisense siRNA templates are transcribed for 2 hours in separate reactions. The reactions are then mixed, and the combined reaction is incubated overnight. Transcribing the templates separately eliminates potential competition between templates for transcription reagents that might limit the synthesis of 1 of the 2 strands of the siRNA duplex. Mixing the transcription reactions facilitates hybridization of the 2 siRNA strands and enables continued RNA synthesis to maximize the dsRNA yield.

**1. Thaw the 2X NTP Mix and 10X T7 Reaction Buffer**

Thaw the 2X NTP Mix and 10X T7 Reaction Buffer at room temperature. After they have thawed, vortex each tube. Check the 10X T7 Reaction Buffer to see if a precipitate is visible, and if so, vortex the tube until the solution is completely resuspended. Briefly spin both tubes prior to using to ensure that no solution is lost when the tubes are opened.

Keep the tube of T7 Enzyme Mix at –20°C and do not vortex it.

**2. Assemble the transcription reactions and mix gently**

a. For each siRNA, assemble 2 transcription reactions at room temperature to synthesize the sense and antisense RNA strands of the siRNA. For each transcription reaction, mix the following components in the order shown:

| Amount | Component |
|---|---|
| 2 µL | sense or antisense siRNA template (from step B.7 on page 11) |
| 4 µL | Nuclease-free Water |
| 10 µL | 2X NTP Mix |
| 2 µL | 10X T7 Reaction Buffer |
| 2 µL | T7 Enzyme Mix |

b. Gently mix contents thoroughly by flicking or brief vortexing and then microfuge briefly to collect the reaction mixture at the bottom of the tube.

**3. Incubate reactions 2 hr at 37°C**

Incubate transcription reactions for 2 hr at 37°C, preferably in a cabinet incubator. (This will prevent condensation, which may occur if the tube is incubated in a heat block.)

**4. Combine the sense and antisense transcription reactions and incubate at 37°C overnight**

Combine the sense and antisense transcription reactions into a single tube and continue incubation at 37°C overnight. The overnight incubation will maximize the yield of RNA and facilitate hybridization of the sense and antisense strands of the siRNA.

## D. siRNA Preparation/Purification

The dsRNA made by in vitro transcription has 5' overhanging leader sequences that must be removed prior to transfection. The leader sequence is digested by a single-strand specific ribonuclease. In the same digestion reaction, the DNA template is eliminated by DNase digestion. The resulting siRNA is recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification. The purified siRNA is eluted from the column into Nuclease-free Water, providing siRNA that is ready for transfection.

**1. Digest the siRNA with RNase and DNase**

a. Thaw the Digestion Buffer at room temperature and vortex the tube to mix the contents thoroughly.

b. To the tube of dsRNA from step C.4 above, add the following reagents in the indicated order:

| Amount | Component |
|---|---|
| 6 µL | Digestion Buffer |
| 48.5 µL | Nuclease-free Water |
| 3 µL | RNase |
| 2.5 µL | DNase |

c. Mix gently, and incubate for 2 hr at 37°C.

**2. Before their first use, add 100% ethanol to the siRNA Binding and Wash Buffers**

Before using the siRNA Binding and Wash Buffers for the first time, add 100% ethanol as shown in the table below, and mix well. The prepared siRNA Binding and Wash Buffers can be stored at room temperature for the life of the kit.

| | Amount of 100% ethanol to add: |
|---|---|
| siRNA Binding Buffer | 5.3 mL |
| siRNA Wash Buffer | 11 mL |

**3. Add 400 µL siRNA Binding Buffer and incubate 2–5 min at room temp**

Add 400 µL of siRNA Binding Buffer to the nuclease digestion reaction and incubate for 2–5 min at room temperature.

**4. Heat Nuclease-free Water to 75°C**

Preheated Nuclease-free Water will be used to elute the siRNA from the Filter Cartridge in step D.7 on page 13.

**5. Prewet a Filter Cartridge with 100 µL siRNA Wash Buffer and bind the siRNA**

a. For each siRNA preparation, place a Filter Cartridge in a 2 mL Tube (provided with the kit).

b. Apply 100 µL of siRNA Wash Buffer to the filter of the Filter Cartridge.

c. Add the siRNA in the siRNA Binding Buffer from step 3 to a prewet Filter Cartridge and spin at ~10,000 rpm in a microcentrifuge for 1 min.

d. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the 2 mL Tube.

**6. Wash the Filter Cartridge with 2 x 500 µL of siRNA Wash Buffer**

a. Apply 500 µL of siRNA Wash Buffer to the filter of the Filter Cartridge and spin at 10,000 rpm for 1 min. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the 2 mL Tube.

b. Repeat the wash with a second 500 µL of siRNA Wash Buffer.

c. Transfer the Filter Cartridge to a new 2 mL Tube.

**7. Elute the siRNA in 100 µL of 75°C Nuclease-free Water**

a. Add 100 µL of the preheated Nuclease-free Water to the filter of the Filter Cartridge and incubate at room temperature for 2 min.

b. Spin the Filter Cartridge at 12,000 rpm for 2 min. The purified siRNA will be in the eluate (in the 2 mL Tube).

**8. Store siRNA at –20°C or –80°C**

siRNAs should be stored at –20°C or –80°C until they are prepared for transfection.

## E. siRNA Quantification

The siRNA concentration used for transfection is critical to the success of gene silencing experiments. Transfecting too much siRNA causes nonspecific reductions in gene expression and toxicity to the transfected cells. Transfecting too little siRNA does not change the expression of the target gene. Assuming that the UV spectrophotometer is accurate, measuring the absorbance of the siRNA sample at 260 nm is the simplest method to assess the concentration of the siRNA preparation.

### 1. Measure the $A_{260}$ of a 1:25 dilution of the siRNA

Dilute a small sample of the siRNA 1:25 into TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and read the absorbance at 260 nm in a spectrophotometer. Be sure to blank the spectrophotometer with the same TE that was used for sample dilution.

### 2. Determine the concentration of the siRNA in μg/mL

Multiply the absorbance reading by 1,000 to determine the concentration of the purified siRNA in μg/mL (explanation below).

1,000 = 25-fold dilution x 40 μg siRNA/mL per absorbance unit

### 3. Determine the molar concentration of the siRNA

The molar concentration of the siRNA in μM can be determined by dividing the μg/mL concentration of the siRNA by 14 (explanation below).

- There are 14 μg of RNA in 1 nmole of an average 21-mer dsRNA:

  21 nt x 2 strands = 42 nt x 0.333 μg/nmol for each nt = 14 μg/nmol

- Dividing the μg/mL concentration by 14 yields the μM concentration as shown below:

$$\frac{\frac{X\,\mu g}{mL}}{\frac{14\,\mu g}{nmol}} \quad \frac{X\,\mu g}{mL} \quad \frac{nmol}{14\,\mu g} \quad \frac{X\,nmol}{mL\,(14)} \quad \frac{X\,\mu mol}{L\,(14)} \quad \frac{X\,\mu M}{14}$$

Therefore μM = X ÷ 14

### 4. Example calculation

A 1:25 dilution of purified siRNA has an $A_{260}$ = 0.4. The molar concentration is determined as follows:

0.4 x 1,000 μg siRNA/mL per $A_{260}$ = 400 μg/mL

400 μg/mL divided by 14 μg siRNA/nmol siRNA = ~29 μM siRNA

## F. Transfecting Mammalian Cells

The efficiency with which mammalian cells are transfected with siRNAs will vary according to cell type and the transfection agent used. This means that the optimal siRNA concentration used for transfections should be determined empirically. We have found that siRNAs generated with the *Silencer* siRNA Construction Kit typically work best when present in cell culture medium at 0.1–10 nM.

Most protocols recommend maintaining mammalian cells in the medium used for transfection. This is to avoid diluting or removing the siRNAs from the cells by adding medium or washing the cells with new medium. We have found that mammalian cells diluted 2 fold with fresh medium 24 hours after transfection typically exhibit greater viability than those left in the medium used for transfection. Furthermore, adding fresh medium does not appear to have a detrimental effect on the activity of the transfected siRNAs.

# III. Troubleshooting

## A. Using the Control DNA Supplied with the Kit

The Sense and Antisense Control DNA templates supplied with the siRNA Construction Kit can be used to generate an siRNA specific to GAPDH. This GAPDH siRNA has been used successfully in human, mouse, and monkey cell lines, thus it can be used both as a control to confirm that the kit is working properly and as a positive control for many siRNA experiments. GAPDH mRNA and protein are expressed at levels in most mammalian cells that can be readily detected by Northern and Western analysis.

### 1. Instructions for the positive control reaction

Use 2 µL of the Sense and Antisense Control DNA in siRNA synthesis following the procedure in this Protocol. Start at step II.B.4 on page 10, and continue to the end of the procedure (finish with step II.D.7 on page 13).

Analyze the outcome of the reaction by measuring the $A_{260}$ of the purified siRNA, and determine the yield in µg as described in step II.E.2 on page 14. Check the size of the siRNA by running 10 µL of the purified siRNA on a 2% agarose gel following the instructions in section IV.A on page 26.

### 2. Expected result of the positive control reaction

The positive control reaction should yield ≥10 µg of siRNA, the majority of which is 21–22 bp. There may also be a minor band on the gel corresponding to an RNA duplex containing 1 incompletely digested 27 nt strand. This secondary band will be ≤30% as intense as the primary band.

### 3. Troubleshooting low yield from the positive control reaction

If the positive control does not yield at least 10 µg of siRNA, then the digestion reaction, column purification, or quantification could be faulty. Distinguish between these 3 possibilities by doing the following experiment.

**a. Reaction setup and analysis (see also Figure 3)**

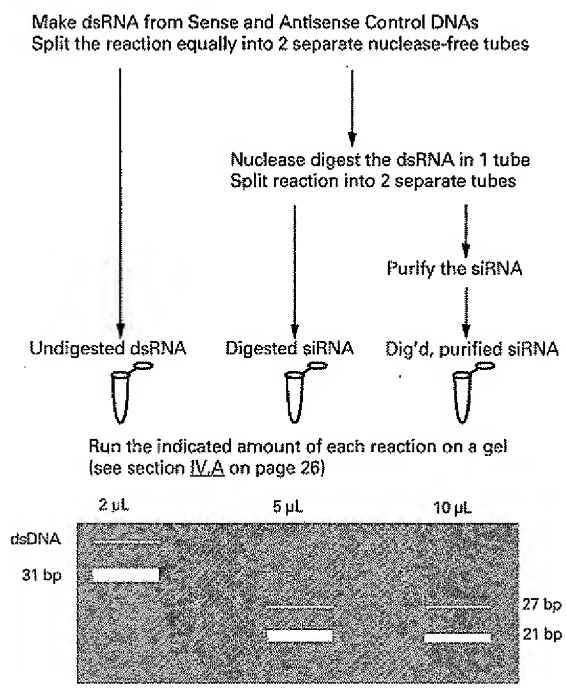
Set up the positive control reaction, and allow it to progress through step II.C.4 on page 12. At this point, split the dsRNA into 2 equal portions (20 µL each) in 2 separate nuclease-free tubes.

i. Set aside 1 tube for gel analysis; this is *undigested dsRNA*.

ii. Nuclease digest the other tube as follows:

| Amount | Component |
|---|---|
| 20 µL | dsRNA |
| 3 µL | Digestion Buffer |
| 24.25 µL | Nuclease-free Water |
| 1.5 µL | RNase |
| 1.25 µL | DNase |

Figure 3. Positive Control Troubleshooting Experiment




iii. Split the digested siRNA into 2 equal portions (25 µL each) in 2 separate nuclease-free tubes.
Set aside 1 tube for gel analysis; this is ***digested siRNA.***
Column purify the contents of the other tube, following the instructions in steps 3–7 on page 13. This is ***digested, purified siRNA.***

iv. Run the amounts indicated below of each reaction on a 2% agarose gel (see section IV.A on page 26).

| Reaction | Amount to run on the gel |
|---|---|
| undigested dsRNA | 2 µL |
| digested siRNA | 5 µL |
| digested, purified siRNA | 10 µL |

**b. Expected result of the positive control troubleshooting reaction**

The undigested reaction should have a primary band at 31 bp. The digested reaction should yield a band at 21 bp that is slightly less intense than the undigested product. The column purified product should provide the same 21 bp product with at least 50% the intensity of the unpurified, digested RNA.

**c. If all 3 reactions generate the expected products and they are easily visible on the gel (but yield in the initial positive control experiment was <10 µg):**

Confirm that the absorbance reading for quantitation was accurate by measuring another RNA whose concentration is known.

**d. If the yield from all 3 reactions is less than expected, then consider the following possible reasons for low yield:**

- The spermidine in the 10X T7 Reaction Buffer may cause precipitation of the template DNA if it is not diluted into a mixture of template, water, and 2X NTP Mix. For the same reason it is important to assemble the transcription reaction at room temperature.
- If you are using a water bath or heat block for the 37°C transcription reaction incubation (steps 3–4 on page 12), then use a cabinet incubator to eliminate condensation that may form during the reaction.
- Check the temperature of the transcription reaction incubation (steps 3–4 on page 12) to confirm that it is 37°C ±2°C.

**e. If the digested siRNA band is less than 50% as intense as the undigested dsRNA, then try the following things to attenuate the nuclease digestion reaction.**

- Reduce the amount of nuclease in the reaction by adding only 25–50% the volume of RNase and DNase to the digestion reaction (step 1 on page 12).
- Reduce the stringency of the digestion reaction (step 1 on page 12) by incubating for 2 hr at room temp rather than at 37°C.

**f. If the digested siRNA band is the expected intensity but the digested and purified siRNA band is less than 50% as intense as the undigested dsRNA band, then try the following things to improve the purification step.**

- Confirm that the siRNA Binding and Wash Buffers were prepared by adding 5.3 mL and 11 mL 100% ethanol respectively before using them the first time (step 2 on page 12). It is also important to mix the buffers well by vortexing or shaking after adding the ethanol.
- Incubate the dsRNA in the siRNA Binding Buffer for at least 2 min before applying it to the Filter Cartridge (step 3 on page 13).

- Elute the siRNA from the Filter Cartridge using 2, 50 µL elutions with Nuclease-free Water instead of a single 100 µL elution (step 7 on page 13).
- During the siRNA elution (step 7 on page 13), apply the Nuclease-free Water to the Filter Cartridge, and incubate the Filter Cartridge for 5 min at 37–50°C before centrifuging.

**g. If none of these suggestions improve the yield of the positive control, then call Ambion's Technical Services Staff for more advice.**

See the back cover of this Protocol for contact information.

## B. Low Yield

*Silencer*® siRNA Construction Kit reactions should routinely yield at least 10 µg of siRNA. Lower yields could result from inefficient transcription of 1 or both of the siRNA templates, overdigestion of the dsRNA preparation, or poor binding or elution from the Filter Cartridge during siRNA purification.

### 1. Do the positive control reaction

Perform the positive control reaction to confirm that the components in the kit are functioning properly (section III.A starting on page 16). If the positive control reaction yields less than 10 µg of RNA, try the troubleshooting experiment described in section III.A.3 on page 16.

### 2. If the positive control reaction works, but your templates give low siRNA yield, try the transcription troubleshooting experiment

If the positive control provides the expected yield but your templates do not, then confirm that both your sense and antisense templates are functioning properly by doing the experiment described below.

**a. Transcription troubleshooting experiment**

i. Using your sense and antisense template oligonucleotides, follow the standard procedure through section II.C, step 3 on page 12. Reaction products from this experiment will be compared to the product of the positive control experiment (described in section III.A on page 16). If you do not have at least 10 µL left from the positive control reaction to run on the gel, do the positive control reaction while doing this experiment.

ii. In step 4 on page 12, mix only 10 µL of the sense transcription reaction with 10 µL of the antisense transcription reaction for the overnight incubation. There will be 3 tubes:

| |
|---|
| 10 µL sense strand reaction |
| 10 µL antisense strand reaction |
| 20 µL reaction for dsRNA |

iii. After the overnight incubation, digest half (10 µL) of the reaction containing both the sense and antisense templates with RNase and DNase as follows:

| Amount | Component |
|---|---|
| 10 µL | dsRNA transcription reaction (containing both sense and antisense template) |
| 1.5 µL | Digestion Buffer |
| 12 µL | Nuclease-free Water |
| 0.75 µL | RNase |
| 0.6 µL | DNase |

iv. Run 4 µL of each of the 3 transcription reactions and 10 µL of the digested siRNA on a 2% agarose gel. Also include a 10 µL sample of the product from the positive control reaction on the gel. (Gel instructions are in section IV.A on page 26.)

**b. Expected result**

The ***sense and antisense reactions*** should generate distinct, 31 nt products with roughly equivalent intensities.

The ***undigested*** transcription reaction containing a ***mixture of the sense and antisense templates*** (undigested dsRNA) should generate a primary product that migrates differently than the single-stranded RNAs. The dsRNA product typically migrates more slowly than the single-stranded products, but some sequences migrate faster when double-stranded.

The ***digested*** transcription reaction containing both templates should generate a product that migrates faster than the undigested dsRNA. Its intensity should be ≥50% the intensity of the undigested dsRNA.

**c. If none of the transcription reactions have good yield:**

If neither of the single template reactions, nor the reaction with both templates generates a robust product (when compared to the positive control reaction), then consider the following:

- Check the sense and antisense templates used for transcription (i.e. the reaction products at step II.B.7 on page 11) using the polyacrylamide gel electrophoresis procedure (see section IV.B on page 26).
  Both templates should migrate as distinct ~50 bp bands. If they do not, then consider gel purifying the template oligonucleotides (see section IV.C on page 27) before using them in the *Silencer* siRNA Construction Kit.

- Confirm that the sequence of the oligonucleotides includes the 8 nt sequence required to hybridize to the T7 Promoter Primer provided with the *Silencer* siRNA Construction Kit (see section II.A.4 on page 8 for instructions on template oligonucleotide design).
- The presence of significant amounts of EDTA or salt can inhibit transcription reactions. If the template oligonucleotides might be contaminated with either, then ethanol precipitate the oligonucleotides and wash the pellets with 70% ethanol before starting the procedure.

**d. If one template generates much less RNA than the other (or none), or if the dsRNA contains a significant amount of ssRNA:**

If one of the templates generates significantly less product than the other in the transcription reactions with a single template, or if the dsRNA product has a significant amount of single-stranded product, then one of the transcription reactions is probably not working properly. This will reduce the yield of dsRNA and subsequently, siRNA. Consider the following suggestions:

- Recheck the concentrations of the template oligonucleotides by measuring their $A_{260}$ and doing the calculations to determine the molar concentration (see step II.B.2 on page 9). The *Silencer* siRNA Construction Kit procedure requires that the template oligonucleotide concentration is 100 µM so that equimolar amounts of template oligonucleotide and T7 Promoter Primer are present in the hybridization step (step II.B.5 on page 10).
- Gel purify the oligonucleotide that generates the less effective template. This treatment could increase transcription yield by increasing the concentration of full-length transcription template, and/or by eliminating inhibitors of transcription from the template.
- If one template doesn't generate any product, confirm the sequence of its template oligonucleotide to make sure it includes the 8 nt sequence required to hybridize to the T7 Promoter Primer (the T7 promoter sequence is required for transcription).
- The presence of significant amounts of EDTA or salt can inhibit transcription reactions. If the template oligonucleotide might be contaminated with either, then ethanol precipitate it and wash the pellet with 70% ethanol before starting the procedure.

- Increase the duration of the transcription reaction in separate tubes (step 3 on page 12) to overnight at 37°C, then combine the sense and antisense transcription reactions and incubate the mixture 1 hr at 42°C instead of at 37°C. Elevating the temperature of the hybridization increases the liklihood that RNA:DNA template hybrids will come apart so that RNA:RNA hybrids can form.

**e. All transcription reactions generate a robust product, but the digested dsRNA is not 21 bp, or it is <50% as intense as the undigested dsRNA:**

If the sense and antisense transcription reactions as well as the combined transcription reaction generate robust products, but the digested dsRNA is not 21 bp, or it generates a band that is less than 50% as intense as the signal seen with the undigested dsRNA, then consider the following:

- Confirm that the sequences for the sense and antisense templates encode fully complementary RNAs.
- Reduce the amount of nuclease in the reaction by adding only 25–50% the volume of RNase and DNase to the digestion reaction (step 1 on page 12).
- Reduce the stringency of the nuclease digestion reaction (step 1 on page 12) by incubating for 2 hr at room temperature rather than at 37°C.

## C. Transfected siRNA Does Not Reduce Gene Expression

Many researchers who are using siRNA have observed that some siRNAs simply do not reduce gene expression. Currently it is not clear why some siRNAs cause gene silencing and others do not. To troubleshoot siRNA that does not reduce gene expression, first check the siRNA on a gel to make sure that it was synthesized properly, then troubleshoot transfection. If the siRNA looks as expected on a gel and options for optimizing transfection are exhausted, but the siRNA still does not reduce gene expression, redesign the siRNA.

### 1. Run the siRNA on an agarose gel to see if it is the expected size

See section *IV.A. Gel Analysis of siRNA* on page 26 for instructions and expected results.

If the siRNA does not have the expected appearance on a gel, see troubleshooting tips in section *III.D. Gel Analysis Shows Multiple Bands or Incorrect siRNA Size* starting on page 23.

**2. Troubleshoot transfection**

Although this is not intended to be an exhaustive list of troubleshooting tips for transfection, it does provide solutions for some of the common problems associated with siRNA experiments in mammalian cells. For more thorough information, please refer to the provider of your transfection agent.

**a. Cell toxicity or nonspecific reduction in RNA or protein levels**

- Use less siRNA in the transfection.
- Dilute the cultured cells 2 fold with fresh medium 24 hr after transfection.
- Try a different transfection agent or procedure.
- Design an siRNA to a different target sequence.
- Gel purify the siRNA (see section IV.C on page 27).

**b. No reduction in the target RNA or protein levels**

- Increase the amount of siRNA in the transfection.
- Try a different transfection agent or procedure.
- Try an siRNA to a different target sequence.

## D. Gel Analysis Shows Multiple Bands or Incorrect siRNA Size

**1. Expected appearance of siRNA on a 12% nondenaturing polyacrylamide gel**

Using the *Silencer* siRNA Construction Kit, we routinely detect a primary siRNA product that is a 21–22 bp dsRNA and a secondary product that contains a 27 nt RNA in the siRNA duplex. The secondary product is typically less than 30% as intense as the primary product. The secondary product appears to have no detrimental effects on siRNA experiments and is likely to be active in targeting mRNA degradation. dsRNA products that are not present in 1 of these 2 bands could potentially limit the effectiveness of an siRNA experiment.

**2. Troubleshooting multiple bands**

If gel analysis shows multiple bands in addition to the primary and secondary products described above, then consider the following:

**a. Check the templates.**

Using polyacrylamide gel electrophoresis (see section IV.B on page 26), check the sense and antisense siRNA transcription templates, i.e. the reaction products at step 7 on page 11, to confirm that there is only a single distinct template band. If it turns out that there are multiple bands in the template, then consider gel purifying the template oligonucleotides used to prepare the transcription templates (see section IV.C on page 27).

**b. Multiple bands can result from ineffective nuclease digestion.**

- If the product bands are primarily larger than the expected 21–22-mer dsRNA, then increase the time of the nuclease digestion reaction (step 1 on page 12) to 4–16 hr or increase the amount of RNase in the reaction from 3 µL to ≤6 µL.

- If the product bands are primarily smaller than the expected 21–22-mer dsRNA, then check the sequences of the sense and antisense template oligonucleotides to confirm that they are complementary. You can also consider incubating the nuclease digestion reaction (step 1 on page 12) at room temperature instead of 37°C, or decrease the amount of RNase in the reaction from 3 µL to ≥1 µL.

**c. If enough of the dsRNA is the correct length, you can gel purify it to generate a pure sample of siRNA.**

See instructions in section IV.C on page 27.

### 3. Troubleshooting siRNA that looks too large on a gel

If gel analysis reveals a single band that migrates as though it is larger than a 21 bp dsRNA, consider the following:

**a. There is a problem with the nuclease digestion**

If there is a single band, and it is the same size as the undigested dsRNA, then the nuclease digestion is not functioning. Confirm that the nuclease digestion reaction (step 1 on page 12) is being incubated at 37°C.

**b. Gel mobility comparison is not always an accurate way to estimate siRNA size**

The gel mobility of siRNA is highly dependent on its nucleotide composition. Because of this, the gel migration of siRNAs with different sequences cannot reliably be compared to determine size. A better standard to estimate the size of an siRNA is the undigested dsRNA from which it is derived.

### 4. Troubleshooting siRNA that looks too small on a gel

If gel analysis reveals a single band that migrates as though it is smaller than a 21 bp dsRNA, consider the following:

**a. Gel mobility comparison is not always an accurate way to estimate siRNA size**

The gel mobility of siRNA is highly dependent on its nucleotide composition. Because of this, the gel migration of siRNAs with different sequences cannot reliably be compared to determine size. A better standard to estimate the size of an siRNA is the undigested dsRNA from which it is derived.

**b. Check the size of the template oligonucleotides and siRNA transcripts**

Confirm that the sense and antisense template oligonucleotides are 29 nt, and that the individual siRNA transcripts are approximately 31 nt by running them on a polyacrylamide gel (you would need to reserve an aliquot of the sense and antisense transcripts from step II.C.3 on page 12 before combining them). A shorter than expected template oligonucleotide or a sequence in the template that disrupts T7 RNA polymerase could create smaller than expected siRNAs. If the template oligonucleotide is smaller than expected, then

have it resynthesized. If the template is the correct size but either or both of the transcripts is smaller than expected, then design oligonucleotides to a different target sequence.

c. **Confirm that the sense and antisense oligonucleotides are entirely complementary.**

A single base mismatch could create a cleavage site for the RNase used in the digestion reaction, resulting in a smaller than expected siRNA.

# IV. Additional Procedures

## A. Gel Analysis of siRNA

siRNA should be assessed by gel electrophoreisis on 2% agarose in TBE (see section IV.A on page 26 for instructions on pouring a 2% agarose gel).

**Instructions for gel electrophoresis**

1. Mix up to 10 µL of siRNA sample with 2 µL of a native gel loading buffer (see a typical recipe in section E.4 on page 29).
2. Load the sample on a 2% agarose gel and electrophorese at about 5–10 mAmps/cm.
3. Stop electrophoresis when the bromophenol blue dye front has migrated two-thirds of the way down the gel.
4. Stain the gel for ~10 min in a 1 µg/mL solution of ethidium bromide.
5. Visualize the siRNA using a UV transilluminator.

**Expected appearance of the siRNA on the gel**

The siRNA should migrate as a 21–22 bp band that runs slightly behind the bromophenol blue dye front. A second, less intense band may be apparent running behind the primary siRNA band. This band represents a dsRNA where the leader sequence of 1 of the strands of siRNA was only partially digested. The underdigested RNA strand is 27 nt and does not create any nonspecific effects when transfected into cells.

## B. Gel Analysis of DNA Oligonucleotides

DNA oligonucleotides can be assessed by gel electrophoresis using a nondenaturing 12% polyacrylamide gel (see section IV.E.3 on page 29) for a 12% polyacrylamide gel recipe).

**Instructions for gel electrophoresis**

1. Mix up to 5 µL of oligonucleotide with 2 µL of a native gel loading buffer (see a typical recipe in section E.4 on page 29).
2. Load the sample on a nondenaturing 12% polyacrylamide gel and electrophorese at 200–250 V.
3. Stop electrophoresis when the bromophenol blue dye front has migrated two-thirds of the way down the gel.
4. Stain the gel for 2–5 min in a 1 µg/mL solution of ethidium bromide.
5. Soak the gel for 2–5 min in water.
6. Visualize the siRNA using a UV transilluminator.

## C. Gel Purification of Nucleic Acids

Gel purification can be used to prepare oligonucleotides or siRNA of a single, defined length. Both siRNA and DNA oligonucleotides should be fractionated using a 12% nondenaturing acrylamide gel (see section E.3 on page 29 for a recipe). For this application, it is useful to have a "preparative scale" comb with teeth about 1–2 cm wide that will form large capacity wells.

### 1. Fractionate the nucleic acids using a polyacrylamide gel

To prepare *siRNA* for electrophoresis, add 20 µL of native gel loading buffer (e.g. see a typical recipe in section E.4 on page 29) to the siRNA following the nuclease digestion step (step II.D.1 on page 12).

For ***oligonucleotides***, mix 1 volume native gel loading buffer (e.g. as in section E.4 on page 29) with 2–4 volumes of oligonucleotide.

Load the entire sample(s) into the freshly-rinsed well(s) of the 12% polyacrylamide gel and run for about 20 min–1 hr at 100–300 volts until the bromophenol blue is approximately 2/3rd of the way to the bottom of the gel.

### 2. Isolate the gel fragment containing the siRNA or DNA oligonucleotide

Nucleic acids can be visualized by UV shadowing. UV shadowing works best if the gel is removed from both glass plates and enclosed in thin plastic wrap. The wrapped gel should then be laid on a Fluor-coated TLC plate (Ambion Cat #AM10110). It may alternatively be possible to use an intensifying screen for visualizing the band, but it will be less sensitive than a Fluor-coated TLC plate.

To see the nucleic acid, direct short wave UV light onto the gel surface in the dark. There must be at least ~400 ng of siRNA or oligonucleotide present in the band to use UV shadowing. Nucleic acids will appear as a dark purple or black band. The xylene cyanol and bromophenol blue bands will also be visible when the gel is illuminated with UV light; if the band of interest comigrates with 1 of these bands, it may be difficult to distinguish between nucleic acid and dye (consider running a lane of loading buffer alone in order to differentiate the dyes from the band of interest).

The siRNA or correct oligonucleotide is usually the most intense band on the gel; it should be excised with a clean scalpel and transferred to about 350 µL of an RNase-free solution in an RNase-free microfuge tube. DEPC-treated water can be used for the elution, however, we recommend using 0.5 M ammonium acetate/ 1 mM EDTA/ 0.2% SDS (Probe Elution Buffer that is included in Ambion's family of nuclease protection assay kits). The EDTA and SDS will inactivate low levels of nuclease and the salt will precipitate the siRNA or oligonucleotide when 3 volumes of 100% ethanol is added.

**3. Elution of siRNA or DNA oligonucleotides from acrylamide gel slices**

To elute the nucleic acids, incubate the gel slice in solution at 37°C overnight. Remove the gel slice from the elution solution and precipitate the siRNA or DNA oligonucleotide with 3 volumes of 100% ethanol. If the elution solution contained less than 300 mM $Na^+$ or $NH_4^+$ ions, then add 1/10th volume of 5 M ammonium acetate or sodium acetate to the elution solution before adding the ethanol. Incubate the precipitation reaction at –20°C for 15 min. Pellet the nucleic acids by centrifuging at 13,200 rpm for 15 min. Aspirate the ethanol and wash the pellet with 70% ethanol. Both siRNAs and DNA oligonucleotides can be dissolved in nuclease-free water and stored at –20°C until they are needed.

## D. Assessing Mammalian Cells Following siRNA Transfection

Cells transfected with effective siRNAs exhibit a reduction in the amount of the targeted mRNA and the protein that it encodes. To assess whether siRNA-mediated gene silencing is occurring, levels of the target RNA or the target protein can be monitored. Ambion offers a comprehensive line of products for both RNA isolation and analysis. Please see section *I.D. Related Products Available from Applied Biosystems* on page 5 for a partial listing of Ambion products that can be used to assess gene silencing in cells transfected with siRNA made with this kit.

## E. Additional Recipes

**1. 10X TBE**

TBE is generally used at 1X final concentration for preparing gels and/or for gel running buffer.

**IMPORTANT**

*Do not treat TBE with diethylpyrocarbonate (DEPC).*

| Concentration | Component | for 1 L |
|---|---|---|
| 0.9 M | Tris base | 109 g |
| 0.9 M | Boric Acid | 55 g |
| 20 mM | 0.5 M EDTA solution | 40 mL |

Dissolve with stirring in about 850 mL nuclease-free water. Adjust the final volume to 1 L.

Alternatively, Ambion offers nuclease-free solutions of 10X TBE (P/N AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (P/N AM9864). Both are made from of ultrapure molecular biology grade reagents.

**2. 2% Agarose Gel**

- Melt 2 g of agarose in 90 mL of water to make 100 mL of gel solution. Typically this is done by putting the mixture in a flask and heating in a microwave. Swirl the mixture in the flask well as the agarose melts to obtain a uniform solution.
- Allow the gel mixture to cool to ~60°C, add 10 mL 10X TBE (for a final concentration of 1X), and swirl to mix well.
- Pour the gel solution into the mold, place the comb, and allow the gel to solidify.
- Place the gel in the gel tank and cover with 1X TBE, then remove the comb carefully.

**3. 12% Acrylamide Gel**

(for 15 mL, enough for a 13 cm x 15 cm x 0.75 mm thick gel):

| Amount | Component |
|---|---|
| 1.5 mL | 10X TBE |
| 4.5 mL | 40% acrylamide (acrylamide: bis acrylamide = 19:1) |
| 9 mL | water (double distilled, deionized) |
| Stir to mix, then add: | |
| 150 µL | 10% ammonium persulfate |
| 15 µL | TEMED |
| Mix briefly after adding the last 2 ingredients, and pour gel immediately. | |

**4. Nondenaturing gel loading buffer**

| Amount | Component |
|---|---|
| 50 % | sucrose |
| 0.25 % | bromophenol blue |
| 0.25 % | xylene cyanol |

**5. RNase-free water**

a. Add DEPC to 0.05% to double-distilled, deionized water (i.e. add 0.5 mL per liter of water).

b. Stir well, incubate several hours to overnight at 37°C or 42°C.

c. Autoclave 2 L or smaller volumes for at least 45 min. The scent of DEPC should be either not detectable or only very slightly detectable.

# V. Appendix

## A. References

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## B. Quality Control

### Functional Analysis

Each component is tested functionally by producing siRNA following the procedure in section II of this Protocol. The reactions should generate >10 µg of siRNA and the majority of the product should migrate as a dsRNA 21–23-mer as analyzed by 20% nondenaturing PAGE.

### Nuclease testing

Relevant kit components are tested in the following nuclease assays:

**RNase activity**
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

**Nonspecific endonuclease activity**
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

**Exonuclease activity**
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

### Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

## C. Safety Information

**Chemical safety guidelines**

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

**About MSDSs**

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

**Obtaining the MSDS**

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.